Original Article:
In Vitro Effect of Pomalidomide on Bone Marrow Mononuclear Cells From Multiple Myeloma Patients

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ABSTRACT

Background: Many features of anticancer drugs, including cytotoxicity and/or cytokine induction, are studied using cell lines or human blood leukocytes. However, in a disease such as multiple myeloma, most cancerous cells are resided within bone marrow mononuclear cells. In the present study, we investigated the effect of pomalidomide on apoptosis and IL-2 production of bone marrow mononuclear cells in patients with multiple myeloma and controls.

Materials and Methods: The Bone Marrow Mononuclear Cells from 10 multiple myeloma patients and 10 controls were cultured using pomalidomide for 48-hour. Apoptosis induction rate, viability, cytotoxicity, and IL-2 production were evaluated.

Results: The results showed that apoptosis and cytotoxicity significantly increased in bone marrow mononuclear cells of multiple myeloma patients cultured in the presence of pomalidomide ($P<0.05$). However, in the control group, pomalidomide led to a slight increase in the vital activity of bone marrow mononuclear cells. Moreover, the concentration of Interleukin-2 (IL-2) in the presence of pomalidomide increased significantly only in the multiple myeloma group ($P<0.05$).

Conclusion: The results of the current study confirmed the toxic effect of pomalidomide on bone marrow mononuclear cells of patients, with no toxic effect on controls. On the other hand, pomalidomide has the potential to increase IL2 production within bone marrow mononuclear cells and consequently make changes in the immune response, which requires further studies for more clarifications.

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Introduction

Multiple myeloma has a complex pathophysiology characterized by the accumulation of malignant plasma cells in the bone marrow accompanied by production of monoclonal immunoglobulin or light or heavy chains, resulting in clinical manifestation of the disease [1-5]. Clinically, multiple myeloma manifests by osteolysis and bone pain, impaired immune system, hypercalcemia, peripheral neuropathy, and renal insufficiency [6-8]. The major problem in cancer treatment is severe damage to healthy tissue in the tumor environment. On the other hand, multiple myeloma comprises 13.4% of all hematologic cancers [4, 9, 10]. It is the seventh most common cancer, according to the cancer registry [11]. It is therefore clear that an effective drug that acts selectively or more potently on cancer cells to stop their growth is a more appropriate choice [12-14].

Novel biological agents targeting tumor cells, tumor cell-host interactions, cytokines, and the BM microenvironment may affect mechanisms of both tumor cell growth and immune responses [15, 16]. For example, Immunomodulatory Drugs (IMiDs) have been used to overcome conventional drug resistance and improve patient outcomes in MM. IMiDs are thalidomide analogues, including lenalidomide and pomalidomide, which possess pleiotropic anti-myeloma properties such as immune-modulation, anti-angiogenic, anti-inflammatory, and anti-proliferative effects [10, 15, 17, 18]. IMiDs also stimulate T-cell proliferation and increase IL-2 and IFNγ production against various myeloma cell lines [19] and also enhance CTL and NK cell activity against these cells [19]. Previous generations of this group, i.e. thalidomide and lenalidomide, have had toxic effects on both normal and cancer cells, although they had powerful anti-angiogenesis and teratogenic properties, as well [21-23].

Pomalidomide is the newest drug of this group, that can increase IL-2 concentration in many myeloma cell lines [24-25] and has a heightened anti-proliferative potency compared with lenalidomide or thalidomide [25-27] But most studies on the effects of pomalidomide are conducted on peripheral blood mononuclear cells or myeloma cell lines [3, 24, 28-30]. Due to the importance of cancer cells accumulation in bone marrow of multiple myeloma patients, it seems appropriate to further study the effects of pomalidomide on bone marrow mononuclear cells, especially in multiple myeloma patients. It was previously reported that pomalidomide does not have toxic effects on normal (or non-cancerous) bone marrow cells [31]. In the present study, we investigated the cytotoxicity and apoptosis vs. survival and IL-2 production in BMMC samples from multiple myeloma and healthy individuals.

Materials and Methods

Patients and control participants

Ethical approval was obtained from the Human Ethics Committee of Shahed University prior to the study. Patients group were selected based on pathological findings (More than 10% plasma cells in bone marrow samples), clinical symptoms, and laboratory data. Clinical symptoms included anemia, weight loss, and bone pain. Patients did not consume any immunosuppressive or anticancer drug and had no history of immunodeficiency. The individuals who were clinically introduced for bone marrow examination but were finally diagnosed with no serious pathologic conditions were considered as control participants.

Cell preparation and culturing

Heparinized aspirates of bone marrow (2 ml) from 10 patients with untreated multiple myeloma and from the control group (10 individuals) were obtained after obtaining the written informed consent. BMMCs were separated by ficoll-hypaque 1077 (Sigma, Germany) density gradient centrifugation. The separated cells were washed three times using culture medium RPMI 1640 (Gibco GlutaMAX) and finally suspended in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mML-glutamine.

Pomalidomide (Sigma P0018, Germany) was dissolved in 0.02% Dimethyl Sulphoxide (DMSO) and stored at -20ºC. 1.5×105 cells/well from each patient and control samples (5 wells for each) were cultured in 96-well plates (JET Biofil, Korea). Next, these cells were cultured in the absence or presence of pomalidomide at 10µM for 48 hour [24]. Cell number and viability were determined using trypan blue and acridine orange/ethidium bromide staining (All from Sigma, Germany).

Light microscopic analysis of cell death

Cells were analyzed using two methods: 1. Giemsa staining (Merck, Germany) of methanol-fixed cytospin (Shandon cytospin 4) preparations were performed. Cells were examined to identify the type of cell death based on the following morphological criteria: Apoptotic cells: nuclear shrinkage and chromatin condensation, cytoplasmic membrane blebbing, reduction of cell volume, and apoptotic body formation; and Necrotic cells: nuclear and cytoplasmic swelling, chromatin flocculation, and

cytoplasmic and nuclear membrane dissolution or lysis; and 2. Trypan blue staining and haemocytometry. In order to estimate the percentage of dead cells, staining with 0.4% trypan blue in PBS was used. After 10 minutes of incubation, the cells (minimum 300) were scored for dye uptake (blue cells are dead).

Fluorescence microscopic analysis of cell death

Acridine Orange and Ethidium Bromide (AO/EB) double staining: acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into Double Stranded Nucleic Acid (DNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. One micro liter (µl) of dye mixture (100 mg/ml AO and 100 mg/ml EB in distilled water) was mixed with 9µl of cell suspension on a clean microscope slide [31, 32]. The suspension was immediately (fast uptake) examined by fluorescence microscopy (Olympus BX51) at 40X magnification. A minimum of 300 cells was counted in every sample.

Cytotoxicity assay

The assay is based on the measurement of Lactate Dehydrogenase (LDH) released into the culture media when the integrity of the cell membrane is affected. The LDH kit (Roche, Germany) also recommends appropriate controls and methods to determine parameters such as percentage cytotoxicity of the cells. All samples (patients and controls) were tested immediately by mixing the media with the assay reagent, prepared by mixing two separate solutions and incubated for 30 minutes, protected from light, and the absorbance was then read at 490 nm, with a reference reading at a wavelength above 600 nm. Furthermore, a control was used consisting of a well into which 10% triton 100X media was added just prior to harvest. This serves as an excellent positive control (for cell lysis).

Cytotoxicity percentage=(experimental value-low control/ high control-low control)×100

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenltetrazolium (MTT) assay

Cells were grown in 96-well plates for 48 hour. Then, at the end of the incubation period, 20µl of MTT (Sigma, Germany) was added to the wells, and the plates were incubated for 3-4 hour at 37°C. After this incubation period, the medium was carefully removed in order to leave the formazan crystals behind. The plates were centrifuged for 5 minutes at 1000 rpm. Then, most of the medium was aspirated off leaving 10-20µl per well and 100µl of DMSO was added to each well to dissolve the formazan crystals. The plates were then agitated for 10 minutes on a plate shaker and the Optical Density (OD) was read immediately on a plate reader at 492 nm.

Index of Viability=(average OD test / average OD control)×100

Interleukin-2 measurement

Levels of IL-2 in culture supernatants were assayed using ELISA (R&D, UK) after 48 hours of incubation at 37°C. Next, 100 µl of primary antibody (anti IL-2) was added to each 96-well plate and incubated at room temperature overnight. The plate was washed filling the wells with 0.5% Tween-PBS buffers. Totally, 100 µl of supernatant samples and diluted standards, then 100 µl of peroxidase-conjugated antibody (secondary antibody), and then 100 µl of chromogenic substrate were added to each well. The reaction was stopped by adding 100µl H2SO4 (0.5 M) to each well and the absorbance was read at 450 nm on an ELISA plate reader.

Statistical analysis

Statistical analyses were performed using commercially available Graph pad Prism software (Version 5.0). Fisher's test was used for nonparametric data. $P<0.05$ was considered statistically significant.

Results

Demographic, clinical and laboratory characteristics in multiple myeloma, and control participants

There were 7 males and 3 females in the patient group and 8 males and 2 females in controls. The mean age of patients and the controls were 70±8 and 64±8 years, respectively. The percentage of bone marrow plasma cells in patients was more than 10% (ranging 10 – 15%); however, in controls, the range was between 3 to 5%. Clinical manifestations were evaluated in both control and patient groups based on medical records review. Bone pain, weight loss, and proteinuria were statistically more frequent in the patient group compared with those in the control group ($P<0.05$) (Table 1).

The average values of biochemical tests including serum creatinine, urea, Erythrocyte Sedimentation Rate (ESR), percentage of gamma bands in protein electrophoresis, and percentage of plasma cells were increased significantly in the patients group compared with those of the control group ($P<0.05$) (Table 3). No significant difference was observed in serum calcium between the two groups ($P>0.05$) (Table 2).

Effect of pomalidomide on induction of apoptosis in multiple myeloma patients

Giemsa staining

To evaluate apoptosis after 48-hour incubation, the cells were spread on glass slides and Giemsa stained. Apoptotic cells were counted according to phenotypic markers by light microscopy. Apoptosis in the patient group increased significantly in the presence of pomalidomide compared with when it was absent (P<0.05) (Figure 1).

Fluorescent staining (AO/EB)

To assess and confirm the apoptosis, BMMCs were mixed with fluorescent dyes (AO/EB) and the percentage of apoptotic cells was calculated by fluorescence microscopy. We distinguished four types of cells accord-

Table 1. Common clinical characteristics in multiple myeloma and control group

Characteristics	Groups		P
	Patient	Control	
Anemia	8.10	6.10	0.62
Bone pain	10.10	3.10	0.003
Weight loss	6.10	1.10	0.05
Proteinuria	6.10	1.10	0.05
Plasma cells%	11±0.02	4±0.01	0.000

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Table 2. Comparison of biochemical and dignostic tests between multiple myeloma patients and control group

Test		Creatinine		Calcium	Urea
		Normal range=0.6-1.2 mg/dl		Normal range=8-10.4 mg/ml	Normal range=15–45 mg/dl
Biochemical tests	Mean±SD	Control group	1±0.16	9.02±0.32	30.1±8.7
		Patient group	1.5±0.43	9.06±0.37	50.2±3.2
	P	0.01		0.86	0.02

Test		ESR		Gamma Band%	Plasma Cells%
		Normal range <20mm		Normal range (15-25)	Normal range (0-5)
Special tests used for multiple myeloma diagnosis	Mean±SD	Control group	17.7±5.04	20±2	4±1
		Patient group	34.7±9.5	32±5	12±2
	P	0.01		0.01	<0.003

ESR: Erythrocyte Sedimentation Rate

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Table 3. Pomalidomide cytotoxicity (percent) in bone marrow mononuclear cells of patient and control groups (LDH-release)

Groups	Low Control (Without Pomalidomide)	Experimental Value (With Pomalidomide)	High Control (Triton 100X)	Cytotoxicity%	P*
Control	0.95±0.15	0.92±0.14	1.44±0.2	-4.06±2%	0.5
Patient	0.97±0.26	1.09±0.2	1.44±0.2	16±4%	0.01
P**	N.S.	N.S.	-	0.01	

*P-value calculated with and without pomalidomide in each group.

**P-value calculated between control and patient groups; N.S.=not significant

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Before treatment

After treatment

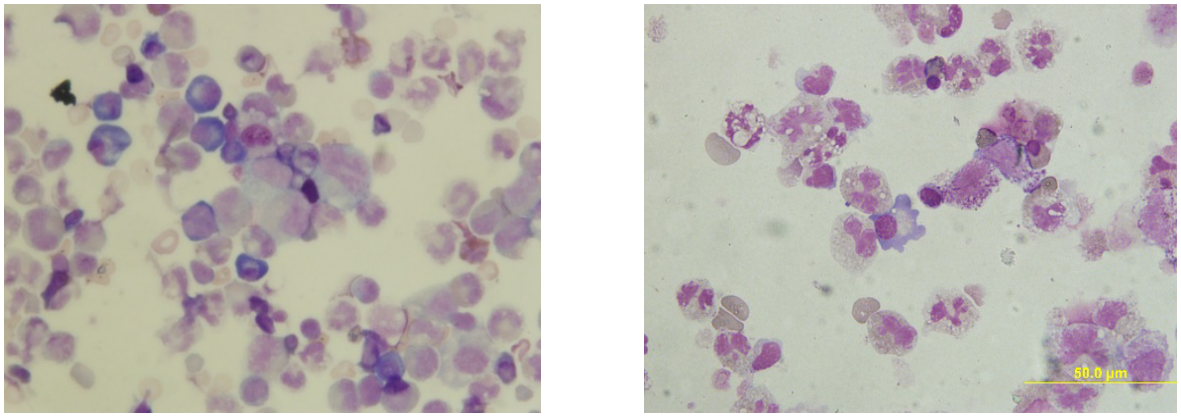

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Figure 1. Induction of apoptosis before and after treatment with pomalidomide in multiple myeloma patients examined by Giemsa staining.

ing to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei: (A)Viable cells have uniform bright green nuclei with organized structure, (B) Early apoptotic cells, which still have intact membranes but have started to undergo DNA cleavage, have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments, (C) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin, and (D) Necrotic cells have a uniformly orange to red nuclei with organized structure (Figure 2).

According to Giemsa and AO/EB staining, apoptosis in cells cultured with pomalidomide in patients was 2-fold higher than cells without the drug, which was statistically significant ($P<0.05$) (Figure 3). However, pomalidomide had no apoptosis induction and the rate of apoptotic cells was not found to be statistically different in the controls ($P>0.05$) (Figure 3).

Effect of pomalidomide on cytotoxicity

The LDH-release assay in culture supernatants was used to study the cytotoxicity in samples with pomalido-

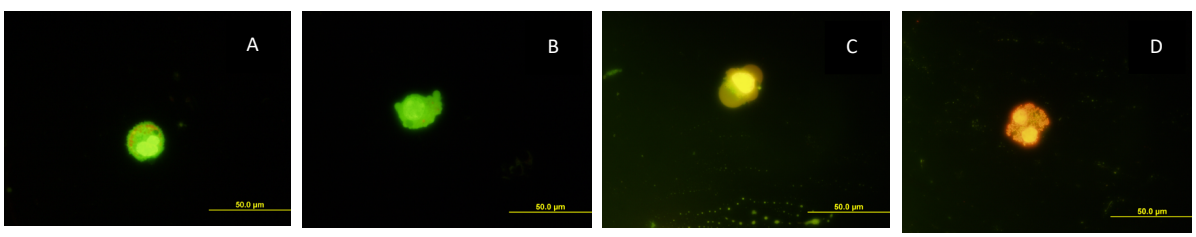

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Figure 2. Patterns of induction of apoptosis in multiple myeloma patients by fluorescent staining according to the morphological aspect of chromatin condensation

(A) Viable cells have uniform bright green nuclei. (B) Early apoptotic cells with bright green patches or fragments. (C) Late apoptotic cells have orange to red nuclei. (D) Necrotic cells have a uniformly orange to red.

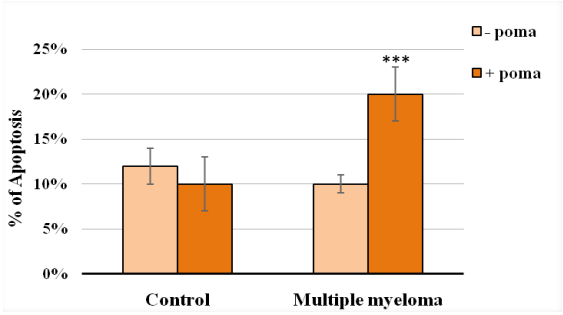

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Figure 3. Effect of pomalidomide on induction of apoptosis (By Giemsa and fluorescent staining): Bone marrow mononuclear cells were separated from 10 multiple myeloma patients and 10 control subjects. 1.5×105 cells/well were cultured with and without pomalidomide in 96-well plates for 48-hour. According to both Giemsa and AO/EB staining, apoptosis in cells cultured with pomalidomide were observed in patients. Asterisks represent statistically significant differences (P<0.05).

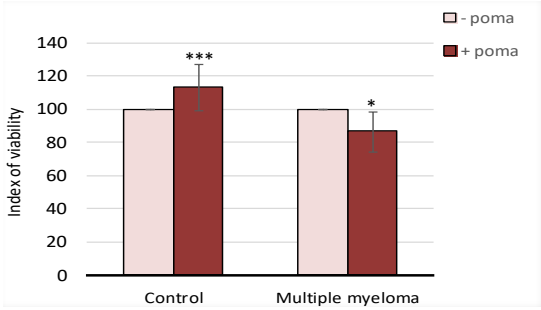

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Figure 4. Effect of pomalidomide on viability

Bone marrow mononuclear cells were separated from 10 multiple myeloma patients and 10 control subjects. 1.5×105 cells/well were cultured with and without pomalidomide in 96-well plates for 48-hour. Then viability of cells was evaluated in each group with MTT assay. Asterisks represent statistically significant differences (P<0.05).

mide (experimental value), without pomalidomide (low control), and with triton 100X, the highest lysis (high control). Then, cytotoxicity was calculated according to the kit formula. The cytotoxicity percentage in the presence of pomalidomide showed a significant increase only in the patient group (P<0.05) (Table 3).

Effect of pomalidomide on viability

To survey the viability of cells in each group, the MTT assay was used and then an index of viability was calculated. The results showed that the index of viability of cells in the presence of pomalidomide had a significant increase (from 100 to 113.5) in the control group (P=0.006). However, in the presence of pomalidomide, this index decreased significantly (from 100 to 86.7) in the patient group (P=0.017) (Figure 4).

Effect of pomalidomide on IL-2 production

The ELISA assay was used to measure IL-2 concentration (pg/ml) in culture supernatants after 48-hour incubation at 37°C. In the patient group, IL-2 production in the presence of pomalidomide increased significantly (P<0.05), but there was no statistically significant difference in the control group (Figure 5).

Discussion

Pomalidomide is one of the newest anticancer drugs named as IMiDs. The previous generations of this group, i.e. thalidomide and lenalidomide, have toxic effects on both normal and cancer cells, although they have powerful anti-angiogenesis and teratogenic properties. [21-23]

Besides, thalidomide has been reported to cause severe birth defects and abnormalities in pregnant women. [19] While pomalidomide induces apoptosis and inhibits angiogenesis much stronger than the previous generation drugs, there are relatively few studies on the effect of pomalidomide on peripheral blood and bone marrow cells. [24, 33, 34] Regarding the importance of cancer cells accumulation in bone marrow of multiple myeloma patients, evaluating the effects of this drug on BMMCs may lead to valuable findings. The present study investigated the toxic and anticancer effects of pomalidomide on bone marrow mononuclear cells of patients with multiple myeloma measuring apoptosis, viability, LDH release, and IL-2 production compared with those in the control participants.

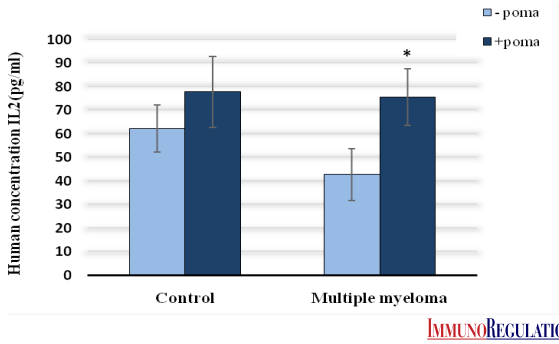

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Figure 5. Effect of pomalidomide on IL2 production (pg/ml)

Bone marrow mononuclear cells were separated from 10 multiple myeloma patients and 10 control subjects. 1.5×105 cells/well were cultured with and without pomalidomide in 96-well plates for 48-hour. Then IL2 concentration in culture supernatants was evaluated in each group with ELISA assay. Asterisk represents statistically significant difference (P<0.05).

LDH is released from the cytosol of dead cells, thus we measured it as an indicator of cytotoxicity. We observed that a significant increase in cytotoxicity occurred in BMMC of patients with pomalidomide, while no toxicity was observed in normal samples. In patients, BMMC is composed of both cancerous and non-cancerous mononuclear cells; therefore, pomalidomide affected the total population of cancerous and non-cancerous cells of bone marrow mononuclear cells. Yet, it can be noticed that there was correlation between cytotoxicity and plasma cell number (myeloid cells). A few studies have reported the toxic effect of IMiDs on tumor cells in BALB/c mice [36] and also myeloma cell lines (e.g. MM.1S, U266) based on the release of radioactive isotopes, such as 51Cr-release and [3H]-thymidine [24, 29]. However, we assayed LDH, which is rapidly released into the cell-culture supernatant and can easily be detected.

The MTT assay indicates the viability and proliferation of cells after cell culture in vitro [33]. In the present study, we calculated viability as the MTT index in BMMC of multiple myeloma patients and showed that it significantly decreased; however, in the control group, a slight increase was observed. It can be argued that the MTT assay complemented and confirmed the results of the LDH test. In addition, we have previously shown that the viability of normal BMMC remarkably increases with pomalidomide in non-cancer participants. [31] In a study of an in vitro erythropoiesis model derived from human CD34+ progenitor cells, the viability of the healthy cells increased in the presence of pomalidomide [33]. Also, the viability of PBMC in myelofibrosis patients improved with pomalidomide treatment [34].

Interleukin-2 is a growth factor and mitogen for T lymphocytes in vitro [37]. No study was found investigating the effects of pomalidomide on IL-2 production in normal bone marrow mononuclear cells. Therefore, in the current study, it was determined that IL-2 production could be elevated with pomalidomide in multiple myeloma patients but not in controls (non-cancerous cells). IL-2 production in the bone marrow plays a significant role in causing cancer cell death. Production of IL-2 from pomalidomide-treated T cells has been shown to be responsible for the enhancement of NK cell activity against cancer cells [36].

In the present study, the basic level of IL-2 in patients was less than that of the controls group, which may be attributed to disorder in IL-2/IL-2R system of multiple myeloma patients [37]. In addition, the immunomodulatory effect of pomalidomide was found to be associated with both innate and adaptive immunity in multiple myeloma. It is recommended that the immunological effects of this drug be studied with more cytokines and effector molecules.

Data collected in the present study showed that pomalidomide eliminated tumor cells in multiple myeloma, with no toxic effects on non-cancerous cells, and may increase hope for effective treatment with pomalidomide with fewer side effects in multiple myeloma. However, it is suggested that the effect of pomalidomide on tumor cells be further studied with more patients and in various conditions. More research is needed to determine pomalidomide functions, both on cancer cells and non-cancer cells in multiple myeloma separately.

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